REMARKS

Claims 45 through 54 are presently pending and under examination. Claim 45 has been amended herein and new claims 55-61 have been added.

Regarding the amendments

Claim 45 has been amended to remove recitation of the phrase "with respect to the function of other receptors."

New claims 55-61 are dependent claims that correspond to base claims 45, 47, 48, 49, 50, 51, and 52, respectively. Each of the newly added dependent claims recites that the conformationally restricted peptide of the corresponding base claim is cyclic. The new claims are supported throughout the specification, for example, at page 8, line 32, to page 9, line 8. No new matter is introduced by the amendment or the

Neither the amendment to claim 45 nor the newly added claims 55-61 introduce new matter. Accordingly, entry of the amendment and new claims is respectfully requested.

Regarding the Rejections under 35 U.S.C. §112, second paragraph

The rejection of claims 45 and 46 under 35 U.S.C. §112, second paragraph, as allegedly indefinite for failing to point out with sufficient particularity the subject matter regarded as the invention, is respectfully traversed. The Examiner asserts

that recitation of the phrase "other receptors" renders the claim indefinite as it allegedly is unclear which other receptors are referred to. Applicants submit that this rejection has been rendered moot by amendment of base claim 45 to omit recitation of the phrase "with respect to the function of other receptors." Accordingly, Appicants respecftully request removal of the rejection of claims 45 and 46 under 35 U.S.C. §112, second paragraph, as allegedly indefinite for failing to point out with sufficient particularity the subject matter regarded as the invention.

Rejections under 35 U.S.C. § 112, First Paragraph

The objection to the specification and corresponding rejection of claims 46, 48, 50, 52 and 54 under 35 U.S.C. § 112, first paragraph, as containing subject matter not described in the specification so as to enable one skilled in the art to practice the claimed invention is respectfully traversed. Applicants respectfully submit that the specification enables the full scope of claims 46, 48, 50, 52 and 54.

The Office Action states that the specification, while enabling for *in vitro* methods of using a conformationally restricted cyclic RGD-containing peptide, does not similarly provide enablement for "*in vivo* methods of use for stabilized stereochemical conformation of a cyclic RGD containing peptide." (current Office Action, Paper No. 8, paragraph bridging pages 2 and 3).

In *Johns Hopkins Univ. v. CellPro, Inc.*, 152 F.3d 1342, 47 U.S.P.Q.2d 1705 (Fed. Cir. 1998), the Federal Circuit clearly stated that routine experimentation does not constitute undue experimentation:

> The test [for undue experimentation] is not merely quantitative, since **a considerable amount of experimentation is permissible**, if it is merely routine, or if the specification in question provides a reasonable amount of guidance with respect to the direction in which the experimentation should proceed to enable the determination of how to practice a desired embodiment of the invention claimed.

Id. (Emphasis added) (citing *PPG Indus., Inc. v. Guardian Indus. Corp.*, 75 F.3d at 1564, 37 U.S.P.Q.2d at 1623); see also *In re Wands*, 858 F.2d at 736-40, 8 U.S.P.Q.2d at 1403-07

Applicants respectfully submit that only routine methods, not requiring undue experimentation, would be necessary for the skilled person to practice the claimed methods *in vivo*. As taught in the specification, the peptide containing the conformationally restricted RGD sequence was effective in inhibiting cell attachment to the vitronectin receptor in rat kidney cell culture (see Example V, pages 13-15). Based on the disclosure of the mammalian cell culture results, the skilled person would have been able to practice the claimed methods in vivo without expenditure of undue experimentation. Applicants respectfully submit that results obtained with living cells derived from an organism and used directly as well as cells grown for multiple generations or indefinitely in culture are

encompassed within and enabling of *in vivo* uses of the claimed methods.

As further evidence of enablement for *in vivo* methods of use for a stabilized stereochemical conformation of a cyclic RGD containing peptide, Applicants submit herewith as Exhibit A, a reference by Tschopp et al., *Current Science* 4:809-817 (1993), which describes *in vivo* methods of use for a conformationally restricted RGD containing peptide. In particular, a cyclic RGD containing peptide was used to inhibit binding of a natural ligand, fibrinogen, to the vitronection receptor, thereby improving thrombolysis and preventing re-thrombosis in a canine model of experimentally induced thrombosis.

As additional evidence of enablement for *in vivo* methods of use for a stabilized stereochemical conformation of a cyclic RGD containing peptide, Applicants submit herewith as Exhibit B, a reference by Ogawa et al., *Nuclear Medicine and Biology* 30: 1-9 (2003), which describes *in vivo* methods of use for a conformationally restricted RGD containing peptide. Thew reference by Ogawa et al. describes use of cyclic RGD containing peptides for the *in vivo* assessment of tumor characteristics based on their binding to the vitronectin receptor, which is turn is expressed on metastatic tumor cells.

Although dated post-filing of Applicants' claimed invention, the above-cited references by those skilled in the art are properly admissible to show that the disclosure was in fact enabling at the time of filing and that only routine

experimentation based on the written description in the disclosure was necessary to practice *in vivo* methods of use for a conformationally restricted RGD containing peptide. See *In re Brana*, 51 F.3d 1560, 1566, 34 USPQ2d 1436, 1441 n.19 (Fed. Cir. 1995)(citing *In re Marzocchi*, 169 USPQ at 370 n.4). The above-cited references corroborate that, based on Applicants' disclosure of the mammalian cell culture results in the specification, the skilled person would have been able to practice the claimed methods *in vivo* without expenditure of undue experimentation.

In view of the above arguments and exhibits, Applicants respectfully submit that, given the guidance provided by the specification, only standard and well-known techniques not requiring undue experimentation, would have been required to practice the invention methods. Accordingly, Applicants respectfullt request removal of the objection to the specification and corresponding rejection of claims 46, 48, 50, 52 and 54 under 35 U.S.C. § 112, first paragraph, as containing subject matter not described in the specification so as to enable one skilled in the art to practice the claimed invention.

Regarding the Rejections under 35 U.S.C. §102(b)

Applicants respectfully traverse the rejection of claims 45, 47, 49, and 51 under 35 U.S.C. §102(b) as allegedly anticipated by Hayman et al., J. Cell Biol. 100: 1948-1954 (1985).

> [A]nticipation requires that the four corners of a single, prior art document describe every element of the claimed invention, either expressly or inherently, such that a person of ordinary skill in the art could practice the invention without undue experimentation.

Advanced Display Sys., Inc. v. Kent State Univ., 212 F.3d 1272, 1282 (Fed.Cir. 2000) (citing Atlas Powder Co. v. Ireco Inc., 190 F.3d 1342, 1347 [Fed.Cir. 1999]; and In re Paulsen, 30 F.3d 1475, 1479 [Fed.Cir. 1994]).

Claim 45 is directed to a method of inhibiting binding of a natural ligand to a vitronectin receptor that encompasses contacting the vitronectin receptor with a peptide containing a conformationally restricted Arg-Gly-Asp sequence, thereby selectively inhibiting binding of the natural ligand to the vitronectin receptor. Hayman et al. does not describe methods utilizing a peptide containing a conformationally restricted Arg-Gly-Asp sequence to selectively inhibit binding of a natural ligand to the vitronectin receptor.

Claim 47 is directed to method of selectively inhibiting attachment of cells to vitronectin by providing to the cells *in vitro* a solution of a peptide containing a conformationally restricted Arg-Gly-Asp sequence, thereby selectively inhibiting attachment of the cells to the vitronectin. Hayman et al. does not describe methods utilizing a peptide containing a conformationally restricted Arg-Gly-Asp

sequence to selectively inhibit attachment of cells to the vitronectin.

Claim 49 is directed to a method of selectively inhibiting binding of vitronectin receptor-containing cells to a substrate by providing to the cells *in vitro* a solution containing a peptide that encompasses a conformationally restricted Arg-Gly-Asp sequence, thereby selectively inhibiting binding of the vitronectin receptor-containing cells to the substrate. Hayman et al. does not describe methods utilizing a peptide containing a conformationally restricted Arg-Gly-Asp sequence to selectively inhibit binding of the vitronectin receptor-containing cells to the substrate.

Claim 51 is directed to a method of selectively inhibiting binding of vitronectin receptor-containing cells to a substrate by the steps of (a) providing to the cells *in vitro* a peptide containing a conformationally restricted sequence Arg-Gly-Asp in solution and (b) contacting the cells with the solution. Hayman et al. does not describe methods utilizing a peptide containing a conformationally restricted Arg-Gly-Asp sequence to selectively inhibit binding of the vitronectin receptor-containing cells to the substrate.

Consequently, Hayman et al. does not disclose all elements of the claimed invention. Accordingly, the rejection of claims 45, 47, 49, and 51 under 35 U.S.C. §102(b) over as allegedly anticipated by Hayman et al. is unsupported by the cited reference and should be removed.

CONCLUSION

In light of the amendments and remarks herein, Applicants submit that the claims are now in condition for allowance and respectfully request a notice to this effect. The Examiner is invited to call the undersigned attorney if there are any questions.

Respectfully submitted,

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Inhibition of coronary artery reocclusion after thrombolysis with an RGD-containing peptide with no significant effect on bleeding time

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Background: A synthetic RGD-containing cyclic peptide, TP9201, specific for the platelet $\alpha_{IIb}\beta_3$ receptor complex, was tested for its ability to accelerate thrombolysis and prevent reocclusion in experimentally induced coronary artery thrombosis.
Methods: Anesthetized, open-chest dogs with occlusive thrombi received tissue plasminogen activator with TP9201 (113 µg/kg bolus; 2.7 µg/kg/min infusion, $n = 7$) or saline control ($n = 9$).
Results: A 2.8-fold increase in the duration of vessel patency from 52.7 ± 63.7 min to 149.1 ± 63.7 min ($P < 0.05$) was observed with TP9201 treatment. The mean duration of vessel occlusion was reduced 2.4-fold from 172.4 ± 81.1 min to 71.7 ± 63.7 min ($P < 0.05$). Administration of TP9201 reduced the mean time to lysis from 76.6 ± 42.9 min to 54.4 ± 42.9 min, but thrombolysis was not significantly accelerated. Persistent patency was observed in four out of seven of the treated dogs compared with none of the nine in the control group ($P < 0.05$). Administration of TP9201 inhibited ex-vivo platelet aggregation stimulated by ADP (30 µM) or collagen (10 µg/ml). No thrombocytopenia or changes in hemodynamic parameters were observed in the treated group compared with the control group. Peptide TP9201 had no effect on bleeding time and the inhibitory effect on ex-vivo platelet aggregation was rapid and reversible. The pharmacodynamic half-life of TP9201 was approximately 1 h with ex-vivo platelet activity returning to baseline within 2 h of discontinuation of treatment.
Conclusions: TP9201 may be an effective therapy for the prevention of re-thrombosis after thrombolytic therapy without adversely affecting hemostasis.

Coronary Artery Disease 1993, **4**:809–817

Keywords: antiplatelet drug, $\alpha_{IIb}\beta_3$ antagonist, thrombolysis, coronary thrombosis, drug-induced bleeding

Either thrombolytic therapy or percutaneous transluminal coronary angioplasty (PTCA) are commonly used to re-establish patency of the coronary artery in the course of thrombotic events. Early treatment of an evolving myocardial infarction with thrombolytic therapy, such as recombinant tissue-type plasminogen activator (rt-PA), has become accepted as a means of limiting infarct size, preserving ventricular function, and reducing mortality [1–4]. Despite the success of thrombolysis in initiating coronary artery reperfusion in 50–80% of patients, acute secondary thrombotic events are common and constitute a major concern [5]. Adjunct therapy with either heparin, aspirin, or dipyridamole (or any two or all three of these adjuncts) reduces the incidence of re-thrombosis to about 20–30%, but no adjunct therapy has been shown to eliminate re-thrombosis [6,7].

A major factor in early artery re-thrombosis appears to be the interaction of platelets with the endothelial surface in the diseased arterial segment [8]. Platelet

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EXHIBIT A

adhesion to normal endothelial surfaces is the initial step in hemostasis and produces a minimal threat of thrombotic occlusion [9]. However, platelet interaction with a diseased endothelial surface leads to platelet adhesion and aggregation resulting in the formation of a platelet-rich, fibrin-containing thrombus that can eventually occlude the vessel.

The platelet integrin $\alpha_{IIb}\beta_3$ (also known as GPIIb/IIIa) plays an important role in both platelet adhesion and aggregation. The final common step of platelet activation by several physiological agonists (i.e. ADP, collagen, thrombin, epinephrine) and shear forces is the binding of fibrinogen to $\alpha_{IIb}\beta_3$, which causes platelet aggregation and subsequent thrombus formation [10]. It has been shown that the tripeptide sequence arginine-glycine-aspartic acid (RGD) is a key recognition domain for the $\alpha_{IIb}\beta_3$ [11,12]. The discovery that RGD-containing synthetic peptides can inhibit fibrinogen binding to $\alpha_{IIb}\beta_3$ suggests that an RGD-containing peptide may be a viable antithrombotic therapy [11–13].

Development of monoclonal antibodies directed against the $\alpha_{IIb}\beta_3$ complex has facilitated the definition of the mechanism of early arterial re-thrombosis [14,15]. F(ab')$_2$ fragments of 7E3, a monoclonal antibody that binds β_3 and inhibits platelet aggregation, have been shown to inhibit platelet-rich thrombus formation, to accelerate coronary thrombolysis, and to prevent reocclusion after thrombolysis [16–20]. However, monoclonal-antibody-derived therapeutic drugs have potential disadvantages: they are immunogenic and they can cause thrombocytopenia and bleeding complications [20,21]. Alternatively, RGD-containing snake venom polypeptides or cyclic RGD-containing peptide derivatives bind to $\alpha_{IIb}\beta_3$ resulting in enhanced and sustained coronary arterial thrombolysis with rt-PA in dogs [22–30]. The disadvantages associated with these peptides include a low degree of specificity for $\alpha_{IIb}\beta_3$ and/or undesired effects on bleeding time *in vivo*.

Recently, the synthetic peptide G4120 was shown to be a potent inhibitor of fibrinogen binding to $\alpha_{IIb}\beta_3$ [31,32]. It also exhibited potent antithrombotic properties in platelet-mediated thrombosis models in the hamster [33] and in the dog [34,35]. However, the intravenous doses required for effective inhibition of in-vivo thrombus formation or of ex-vivo platelet aggregation are associated with a marked prolongation of the template bleeding time [34,35]. By contrast, the $\alpha_{IIb}\beta_3$ inhibitor TP9201, a synthetic cyclic RGD-containing peptide, was found to have a potency for the inhibition of ex-vivo platelet aggregation comparable to that of G4120, but the intravenous doses at which it inhibits ex-vivo platelet aggregation are not associated with prolongation of bleeding time [36,37].

In the present study, we explored the efficacy of an intravenous infusion of the RGD-containing $\alpha_{IIb}\beta_3$ antagonist, TP9201, in increasing initial thrombolytic efficacy and decreasing the occurrence of re-thrombosis. We used an experimental model in which thrombosis was induced by electrical stimulation of the intimal surface of the left circumflex (LCX) coronary artery at the point of an external stenosis [38]. The effect of the administration of TP9201 on the frequency and rate of arterial recanalization and on the prevention of early and late reocclusion, as well as its effect on clot lysis, were evaluated.

Methods

Animal investigation

These studies conform to the 'Position of the American Heart Association on Research Animal Use' (11 November 1984). The procedures followed in this study were in accordance with the guidelines of the University of Michigan (Ann Arbor) Committee on the Use and Care of Animals. Veterinary care was provided by the University of Michigan Unit for Laboratory Animal Medicine. The University of Michigan is accredited by the American Association of Accreditation of Laboratory Animal Care, and the animal care and use program conforms to the standards in 'The Guide for the Care and Use of Laboratory Animals,' Department of Health, Education, and Welfare Publication number NIH 78-23.

Surgical preparation and instrumentation

The surgical preparation and instrumentation of the animals has been described in detail previously [38]. Briefly, mongrel dogs weighing 14–21 kg were anesthetized with sodium pentobarbital (30 mg/kg intravenously), intubated, and ventilated with room air at a tidal volume of 30 ml/kg and a frequency of 12 breaths per minute (Harvard respirator, Harvard Apparatus, South Natick, MA). The left carotid artery and internal jugular vein were exposed, and catheters were inserted to monitor arterial pressure (Statham P23DC Pressure Transducer, Spectra Med, Critical Care Division, Oxnard, CA) and the infusion of drugs, respectively. A left thoracotomy was performed in the fifth intercostal space and the heart was suspended in a pericardial cradle. A 2-cm section of the LCX coronary artery was isolated proximal to the first obtuse marginal branch and instrumented from proximal to distal with an electromagnetic flow probe (Model 501, Carolina Medical Electronics, King, NC), an intracoronary catheter electrode, and an adjustable mechanical occluder.

The stimulation electrode was constructed from a 25-gauge stainless steel hypodermic needle tip attached to 30-gauge Teflon-insulated, silver-coated copper wire. The mechanical constrictor was constructed of stainless steel in a C-shape with a Teflon screw (2 mm diameter), which could be manipulated to control vessel circumference. The constrictor was adjusted to decrease the reactive hyperemic flow (resulting from a 10-second mechanical occlusion) by 30% without affecting mean resting coronary blood flow. The small intervening coronary branches over the 1–2-cm segment were ligated. Throughout the protocol, continuous recordings of the limb lead II ECG, arterial pressure, and mean and phasic coronary artery blood flow were obtained on a Grass model 7 polygraph recorder (Grass Instrument Co., Quincy, MA).

Protocol

Thirty minutes after surgical preparation of the animal, a 100 µA continuous anodal direct current was applied to the luminal surface of the LCX coronary artery via the intracoronary electrode [39]. The anodal direct current was delivered from a 9 V nickel-cadmium battery with the anode connected in series via a 250 000 Ω potentiometer to the intraluminal coronary artery electrode. The electrical circuit was completed by placing the cathode in a subcutaneous site. The anodal current was maintained until the LCX artery blood flow decreased to, and remained at, 0 ml/min for 30 min. The resulting electrolytic lesion of the endothelial surface of the artery produces a thrombogenic environment and ultimately an occlusive thrombus is formed. As this process evolves, oscillations in coronary artery blood flow occur. These oscillations are attributed to enhanced vasomotor activity of the vessel, the formation and dislodgement of partially occlusive platelet thrombi such as those described by Folts *et al.* [40], or a combination of these events. Current was delivered to the electrode for up to 3 h, or was switched off after 30 min of persistent thrombotic LCX coronary artery occlusion, whichever occurred first. Mean LCX coronary artery blood flow was recorded with a calibrated extracorporeal electromagnetic flow probe and was plotted as a function of time during reperfusion. Occlusion was defined as a 0 ml/min LCX coronary artery blood flow, detected by the flow probe, with ST-segment elevation on limb lead II of the ECG. The study was initiated 30 min after thrombotic occlusion of the LCX by a bolus injection of TP9201 (or saline in control animals) immediately followed by an infusion to maintain steady state drug plasma levels for 3 h. Five minutes after the bolus injection, 1 mg/kg rt-PA (Activase) was given as follows: 10% as a bolus and 50% as an infusion during the first hour, 20% as an infusion during the second and third hours. The study was stopped either after the discontinuation of drug administration or, in some animals, after an additional 90 min had passed. The return of blood flow to 10% of baseline or above was considered to indicate reperfusion. Vessel patency was defined as continuous blood flow at 10% baseline flow or above. Cyclic flow variations were considered to be reocclusion events even if subsequent reperfusion occurred. Bleeding time incisions were made using an automated spring loaded device (Simplate-II, General Diagnostics, Morris Plains, N.J.) applied to the surface of the tongue. The heart was electrically fibrillated, excised, rinsed, blotted dry, and weighed. The LCX coronary artery was isolated, dissected free, and the blood clot removed for weighing.

Platelet aggregation and coagulation studies

Blood (20 ml) was withdrawn from the jugular cannula into a plastic syringe containing 3.2% sodium citrate as the anticoagulant (citrate volume to blood volume ratio 1 : 10) at baseline, 15, 75, 135 min and 3 h after treatment with either TP9201, or saline vehicle. The platelet count was determined with a Haema Count MK-4/HC system (J.T. Baker, Allentown, PA). Platelet-rich plasma (PRP), the supernatant present after centrifuging anticoagulated whole blood at 1000 rpm for 5 min (140 *g*), was diluted with platelet-poor plasma (PPP) to achieve a platelet count of 200 000/µl. PPP was prepared after the PRP was removed by centrifuging the remaining blood at 12 000 *g* for 10 min and discarding the bottom cellular layer.

Ex-vivo platelet aggregation was determined by established spectrophotometric methods with a four-channel aggregometer (BioData-PAP-4, BioData Corporation, Hatboro, PA) by recording the increase in light transmission through a stirred suspension of PRP maintained at 37°C. Aggregation was induced with ADP (30 µM) or collagen (10 µg/ml). Values were expressed as a percentage of aggregation. This represents the percentage of light transmission standardized to PRP and PPP samples yielding 0% and 100% light transmission, respectively. Citrate-anticoagulated plasma was used to study the prothrombin time, activated partial thromboplastin time, and the fibrinogen concentration as described previously [20].

Reagents

Single-chain rt-PA was obtained from Genentech, Inc. (South San Francisco, California). The peptide TP9201 (acetyl-L-cysteinyl-L-asparaginyl-L-propyl-L-arginyl-glycyl-L-α-aspartyl-o-methyl-L-tyrosyl-L-arginyl-L-cysteine-amide, cyclic 1 to 9-sulfide) was supplied in a crystalline form by Telios Pharmaceuticals, Inc. (San Diego, California). Peptide TP9201 inhibits platelet aggregation in citrated human plasma with IC_{50} of 0.2 µM and in heparinized human plasma with IC_{50} of 2.7 µM [36,37]. The initial dose of TP9201 (113 µg/kg) was dissolved and administered in 10-ml pyrogen-free 0.9% sodium chloride solution for injection. The infusion dose (2.7 µg/kg/min) was dissolved in the appropriate amount of pyrogen-free sodium chloride solution and administered for 3 h at a rate of 0.4 ml/min immediately after the bolus dose (total dose was 0.6 mg/kg). This dose and administration regimen was selected to maintain complete inhibition of platelet aggregation *ex vivo* during the course of the experiment.

Statistical analyses

The data are expressed as means ± SD. Differences between groups were analyzed using the Student's t-test, when applicable. Time-dependent changes in hemodynamic parameters, platelet counts and aggregations, and coagulation profile were determined by repeated analysis of variance. If a significant interaction was present, the Bonferroni multiple comparison method was used to determine which treatment means differed significantly. The Fisher exact test was used to assess the difference in the incidence of thrombosis and the incidence of cyclic flow oscillations between treatment groups. Differences between groups were considered significant if $P \leq 0.05$.

Results

Group characteristics

Sixteen dogs were studied successfully under the described protocol. After surgical preparation, each animal was assigned randomly to either the rt-PA/saline group (n = 9) or the rt-PA/TP9201 group (n = 7). The mean body weight did not differ between the control (17.4 ± 1.6 kg) and TP9201 (16.5 ± 2.7 kg) groups. The weight of each heart was determined post mortem and there was no difference between the two groups. Baseline measurements of various hemodynamic parameters (heart rate, mean arterial

Table 1. Coronary artery patency status.

Treatment	Persistent patency	Cyclic reflow with reocclusion	Reflow with reocclusion	Occluded at end of drug treatment (180 min)	Occluded at end of experiment
Control, rt-PA	0/9	7/9	2/9	5/9	7/9
TP9201, rt-PA	4/7	3/7	0/7	0/7	1/7
Statistical significance: control versus TP9201	$P = 0.019$	$P = 0.16$	$P = 0.30$	$P = 0.029$	$P = 0.020$

pressure, and LCX coronary artery blood flow) were not different between the groups. Coronary blood flow in the LCX vessel was similar between the two groups at the initiation of the protocol (control, 34.9 ± 14 ml/min versus TP9201 37.3 ± 11.5 ml/min). The time required for occlusive thrombus formation to occur (at which time coronary flow was zero) was the same in control and TP9201 groups. In each of the two groups studied, the LCX coronary artery occluded within 3 h of the application of anodal current to the intimal surface of the artery.

Vessel patency and coronary artery thrombosis

The effect of peptide TP9201 on thrombolysis and re-thrombosis was determined (Fig. 1). There was a slight decrease in the time to thrombolysis in the TP9201-treated animals compared with those treated with saline. The mean time to lysis in the presence of TP9201 was 54.4 ± 42.9 min compared with 76.6 ± 42.5 min in the control group. The difference, however, was not statistically significant ($P = 0.32$). By contrast, the mean time to first reocclusion after initial lysis was significantly increased from 5.0 ± 5.8 min in the control group to 89.1 ± 92.9 min with TP9201 treatment ($P < 0.05$). Peptide treatment resulted in a 2.8-fold increase in the mean duration of vessel patency from 52.7 ± 63.7 min to 149.1 ± 63.7 min compared with control ($P < 0.05$). At the same time, TP9201 treatment reduced the mean duration of vessel occlusion 2.4-fold from 172.4 ± 81.1 min to 71.7 ± 63.7 min ($P < 0.05$). All nine animals in the control group reoccluded after initial thrombolysis, but four out of seven animals in the TP9201-treated group remained persistently patent throughout the course of the experiment (Table 1). Only three out of seven animals in the TP9201-treated group showed cyclic reflow with reocclusion, whereas all nine animals in the control group showed either cyclic reflow with reocclusion (n = 7), or reflow with reocclusion (n = 2). At the end of drug treatment (180 min) none of the seven TP9201-treated animals was occluded compared with five out of nine animals in the control group (Table 1). These results clearly show that treatment with TP9201 improves the overall vessel patency after thrombolysis with rt-PA. In some animals the length of the experiment was extended up to an additional 90 min after drug treatment. At the end of the experiment, six out of seven TP9201-treated animals remained patent compared with two out of nine animals in the control group (Table 1). Overall, there was no difference between the mean duration of the experiment with TP9201 (225 ± 38.6 min) and that of the saline control group (220 ± 44.2 min).

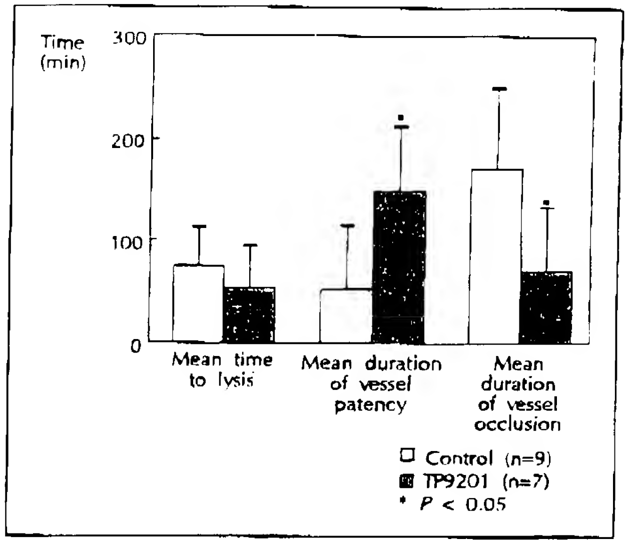

Fig. 1. Thrombolysis and re-thrombosis. Mean time to lysis is the average time from TP9201 or saline administration to first lysis of the thrombus measured by LCX coronary artery blood flow (mean ± SD). Mean duration of patency (mean ± SD) is the average time of LCX coronary artery blood flow and mean duration of occlusion (mean ± SD) is the average time LCX coronary artery blood flow was undetectable for the duration of the experiment. Asterisks indicate significant difference ($P < 0.05$) from the control value.

Thrombus weight

At the conclusion of the experimental protocol, the thrombus was removed from the LCX coronary artery and weighed. A residual thrombus was present in every animal. The mean thrombus weight from the control group was 9.6 ± 7 mg compared with 5.0 ± 2.7 mg from the TP9201 group. The mean thrombus weight from the TP9201-treated animals tended to be less compared with the control group, but the difference was not statistically significant. Although a small thrombus was present in each of the seven animals in the TP9201-treated group, only one

thrombus was occlusive and the remaining six animals had patent coronary arteries at the end of the protocol. By contrast, seven out of nine animals in the control group had occlusive thrombi at the conclusion of the experiment.

Platelet aggregation studies

The platelet aggregatory response to ADP and the platelet counts of the control and TP9201-treated groups were not different at baseline. The data for the platelet aggregation studies during TP9201 treatment are summarized in Table 2. The aggregation response to ADP during treatment with TP9201 was completely inhibited (Table 2), and the same result was found for the aggregation response to collagen (10 μg/ml) or arachidonic acid (0.375 mM)(data not shown). The platelet counts during TP9201 treatment varied and ranged from 396 000 to 494 000/μl, well within the normal range. Platelet counts did not change significantly over the course of the experiment (Table 2). Therefore, the presence of TP9201 did not cause thrombocytopenia.

Hematology and hemodynamic responses

It has been reported in previous in-vivo studies with $\alpha_{IIb}\beta_3$ antagonists that template bleeding time is increased as a result of drug treatment [20,34,35]; therefore, the effect of TP9201 on the template bleeding time at efficacious doses was determined. At the onset of the experiment, template bleeding time was similar between the two study groups (Table 3). During the course of drug treatment, the template bleeding times in some animals of both groups were transiently increased after rt-PA administration. Fluctuations in the bleeding time measurements were detected, but there was no significant increase in the bleeding time of the TP9201-treated group (Table 3). No excessive bleeding or oozing from the surgical wounds of the treated animals was observed. TP9201 had no effect on the prothrombin time, partial thromboplastin time, and fibrinogen concentration as determined in a separate group of animals not treated with rt-PA (data not shown). The mean arterial pressure and heart rate of all animals were recorded continuously throughout the protocol. No significant changes were noted over time within either group and no differences between groups were detected (data not shown).

Pharmacodynamic properties of peptide TP9201

The TP9201 dose used in this experiment group was derived from the results of pharmacodynamic experiments in a separate group of dogs. A bolus injection of 50 μg/kg TP9201 causes immediate ex-vivo inhibition of platelet aggregation in citrated plasma. This effect was sustained by a continuous peptide infusion of 1.2 μg/kg/min (Fig. 2). Thirty to forty minutes after discontinuation of TP9201 administration, ex-vivo platelet activity begins to recover and reaches baseline values within 2–3 h. The pharmacodynamic half-life of TP9201 is estimated to be approximately 1 h and the minimum dose required to maintain effective inhibition of ex-vivo platelet aggregation stimulated by ADP (30 μM) or collagen (10 μg/ml) was determined to be 37 μg/kg bolus followed by continuous infusion of 0.9 μg/kg/min (data not shown). Peptide TP9201 inhibits platelet

Table 2. Ex-vivo ADP-induced platelet aggregation and platelet count during TP9201 treatment.

Timepoint (min)	TP9201/rt-PA treatment: Platelet aggregation (%)	TP9201/rt-PA treatment: Platelet count ($10^3/\mu l$)	Saline/rt-PA treatment: Platelet aggregation (%)	Saline/rt-PA treatment: Platelet count ($10^3/\mu l$)
Baseline	70 ± 18	417 ± 142	63 ± 7	359 ± 99
15	−5 ± 8	451 ± 184	31 ± 10	291 ± 123
75	−2 ± 10	493 ± 320	45 ± 11	357 ± 120
135	−5 ± 11	396 ± 267	ND	289 ± 115
180	−2 ± 7	494 ± 260	24 ± 9	345 ± 145

ND, not determined.

Table 3. Average template bleeding time in control and TP9201-treated animals.

Treatment	Baseline	15 min	75 min	135 min	180 min
Saline, rt-PA	2.8 ± 1 min[c)]	4.1 min[b)]	2.5 ± 0.7 min[c)]	1.8 ± 0.8 min[c)]	1.9 ± 0.5 min[c)]
TP9201, rt-PA	2.1 ± 1 min[c)]	1.5 min[a)]	3 min[b)]	4 min[b)]	3 ± 0.7 min[c)]

[a)] n = 1, [b)] n = 2, [c)] n = 4 to 6. Baseline versus 180 min in both groups not significant ($P > 0.05$).

aggregation in citrated dog plasma with IC_{50} of 0.3 μM and in heparinized dog plasma with IC_{50} of 1.7 μM. These IC_{50} values correspond to 0.34 μg/ml and 1.93 μg/ml plasma, respectively.

Discussion

The present study shows the effect of the RGD-containing cyclic peptide TP9201 on thrombolysis and re-thrombosis in a canine model of experimentally induced coronary artery thrombosis. The electrolytic injury results in endothelial denudation, intimal wall injury, and the formation of an occlusive thrombus at the site of an external constrictor. The external constrictor mimics atherosclerotic narrowing of a coronary vessel [38]. At the site of vessel injury, an occlusive platelet-rich thrombus firmly adheres to the damaged region, thus exhibiting many features similar to clinical thrombotic lesions. We show that TP9201 treatment can inhibit re-thrombosis after thrombolysis and can significantly improve overall coronary artery patency compared with a control group treated with rt-PA alone. Acceleration of thrombolysis in the presence of TP9201 was observed but was not statistically significant. This apparent acceleration of thrombolysis may be a net effect of decreasing time to lysis through inhibition of re-thrombosis during thrombolysis.

Improvement of thrombolysis and inhibition of re-thrombosis has been reported previously with the $\alpha_{IIb}\beta_3$-specific monoclonal antibody 7E3 and with various RGD-containing peptides [16–20,25,26,29,30, 32–35]. Although 7E3 has been shown to be efficacious in several animal models of thrombosis as well as in clinical studies [41], certain drawbacks exist. Monoclonal antibodies are potentially immunogenic even after the replacement of murine sequences with human ones [21]. The inhibitory effect of monoclonal antibodies on platelet aggregation is irreversible and the inhibited platelet population must be replaced before normal hemostasis can be achieved. Monoclonal antibodies that inhibit $\alpha_{IIb}\beta_3$ increase significantly the template bleeding time in animals and in humans, and in some cases cause thrombocytopenia [42]. The high bleeding times are not readily reversible and will only slowly decrease with the formation of new platelets or with infusion of platelet concentrates. Although it has been recently reported that template bleeding time does not correlate with bleeding events in a patient population receiving the 7E3 antibody, the potential problems of surgical bleeding and blood loss caused by irreversible $\alpha_{IIb}\beta_3$ blockage have not been addressed [42]. Small RGD-containing peptides may have certain advantages over $\alpha_{IIb}\beta_3$-inhibiting antibodies because, like many other small peptides, they are fast-acting, reversible, and not immunogenic [43,44].

The relation between antithrombotic effect, ex-vivo platelet aggregation and bleeding time prolongation in the presence of TP9201 was studied. Our observations suggest that TP9201 is efficacious in the present coronary thrombosis model at doses that only partially inhibit ex-vivo platelet aggregation under physiological conditions. The larger IC_{50} value in heparinized blood (which represents a more physiological environment than citrated blood) was found to be a consequence of the calcium concentration and was not caused by the presence of the anticoagulant [36]. This apparent separation of activity under two different conditions appears to be a unique feature of TP9201 not observed with other similar RGD-containing peptides [36]. Other RGD-containing pep-

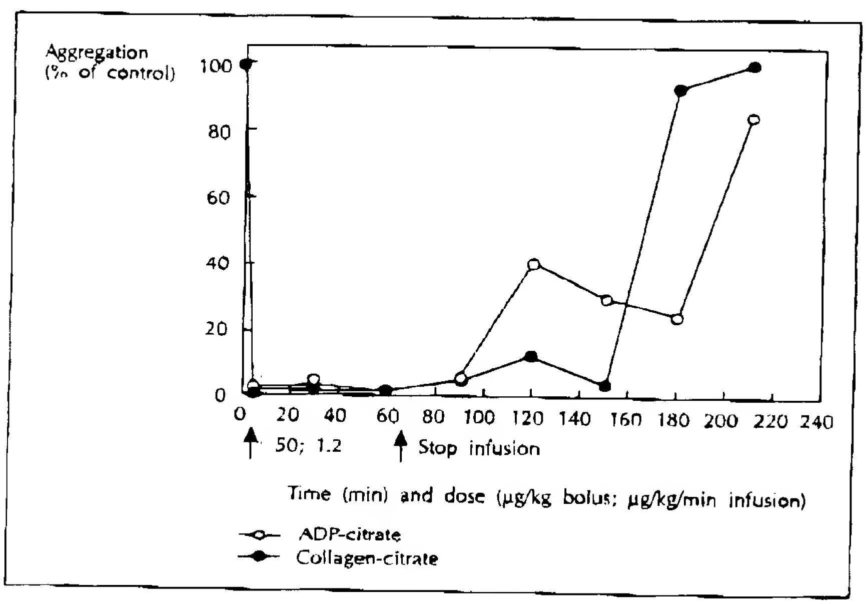

Fig. 2. Canine pharmacodynamics with TP9201. Platelet aggregation (% of control) was determined *ex vivo* in citrated platelet-rich plasma before and after TP9201 bolus (50 μg/kg) administration followed by a 1 h infusion (1.2 μg/kg/min) using ADP (30 μM) or collagen (10 μg/ml) as agonists. After discontinuation of drug administration, ex-vivo platelet aggregation was monitored until platelet activity reached baseline values. Figure shows typical results obtained from one experiment in one animal.

tides have been shown to be efficacious only after substantial inhibition of platelet aggregation has been achieved [34]. In these cases, however, an increase in template bleeding time was observed. It is possible that differences among RGD-containing $\alpha_{IIb}\beta_3$ peptide antagonists exist and that they may differ in their mechanism of action.

Measurement of inhibitory activity in heparinized blood seems to be more relevant to physiological conditions and therefore should predict efficacy. However, TP9201 achieves its antithrombotic effect with moderate inhibition of platelet aggregation in heparinized blood. Peptide TP9201 was found to be efficacious at doses that do not completely inhibit ex-vivo platelet aggregation in heparinized plasma and do not increase the template bleeding time. This result suggests that TP9201 may be used safely over a wide therapeutic dose range. Peptide TP9201 had no effect on prothrombin time, partial thromboplastin time, and fibrinogen concentration, which suggests that it achieves its antithrombotic effect without affecting the coagulation parameters. By contrast, a recombinant form of hirudin (r-hirudin), a specific and potent inhibitor of thrombin that was shown to decrease the incidence of thrombosis in this same thrombosis model, increased the prothrombin time 1.5–2-fold and the partial thromboplastin time 2.5-fold [45]. Ex-vivo platelet aggregation induced by arachidonic acid or collagen was not affected by r-hirudin, which may explain why the drug has only a partial inhibitory effect on the platelet-rich thrombi produced in this model.

In this model, treatment with TP9201 during thrombolysis does not result in the elimination of the thrombus or in a significant reduction of its size. However, inhibition by TP9201 administration of ex-vivo platelet aggregation induced by ADP, collagen, arachidonic acid, or thrombin may explain the observed trend in decreased platelet accumulation onto the surface of the injured vessel, thus inhibiting extensive propagation and formation of an occlusive thrombus in the vessel lumen. A similar reduction of thrombus mass, but not total inhibition of thrombus formation, was observed in the same model during treatment with hirudin [45]. Hirudin treatment, however, did not alter thrombus composition with respect to the number of platelets per milligram of thrombus mass. Platelet aggregation responses to collagen, arachidonic acid, and probably ADP were not altered during hirudin treatment. Therefore, platelet aggregation stimulated by agents other than thrombin may occur and could contribute to intravascular thrombus formation. In this model, it appears that antiplatelet therapy is more effective than antithrombin therapy in preventing coronary artery re-thrombosis and that TP9201 may constitute a preferred agent for the prevention of platelet-mediated arterial thrombosis [46]. The peptide TP9201 may enhance the efficacy of antithrombin therapy; however, the clinical efficacy of antiplatelet therapy versus direct thrombin inhibition with agents such as r-hirudin has yet to be determined. In addition, it is not known what length of treatment is required with either antiplatelet or antithrombin agents to achieve long-term efficacy and elimination of a rebound effect after discontinuation of drug treatment.

Thrombolytic therapy is at present underused in the USA even though it provides substantial reductions in morbidity and mortality rates [47]. This underuse is primarily a result of ineligibility criteria, two of which are advanced age and specific contraindications; both criteria include a perceived increased risk of hemorrhage from thrombolytic therapy. We propose that adjunctive antiplatelet therapy with TP9201 may reduce the risk of hemorrhage associated with thrombolytic therapy because the effective dose of the thrombolytic agent may be decreased, thus reducing the hemorrhagic risk without compromising thrombolysis. Some ineligibility criteria could then be relaxed and more patients could benefit from thrombolytic therapy. Reduction of rethrombosis achieved by TP9201 treatment may also translate into reduction in the need for secondary intervention such as angioplasty or cardiopulmonary bypass, or it may result in an improvement of cardiac function and a reduction in infarct size. Also, the efficacy of thrombolytic therapy in otherwise non-responsive patients may be increased in the presence of this $\alpha_{IIb}\beta_3$ antagonist.

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Direct electrophilic radiofluorination of a cyclic RGD peptide for *in vivo* $\alpha_v\beta_3$ integrin related tumor imaging

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Abstract

The association of the $\alpha_v\beta_3$ integrin with tumor metastasis and tumor related angiogenesis has been suggested. Therefore, by imaging the $\alpha_v\beta_3$ receptor with PET, information concerning the tumor status could be obtained. Cyclic peptides including the RGD sequence, were radiolabeled by direct electrophilic fluorination with [^{18}F]AcOF. In tumor-bearing mice, the labeled peptides accumulated at the tumor with a high tumor to blood ratio. These findings suggest that an assessment of tumor characteristics may be obtained by using these ^{18}F-labeled peptides.

Keywords: Fluorine-18; Radiofluorination; RGD peptide; $\alpha_v\beta_3$ integrin; Tumor imaging

1. Introduction

Integrins are a family of transmembrane glycoproteins with relatively small cytoplasmic domains [24]. They consist of two subunits, α and β, and to date, 25 different integrin $\alpha\beta$ heterodimers have been reported [23]. Integrins function as receptors for extracellular matrix (ECM) proteins or for membrane bound counter-receptors on other cells, such as laminin, fibronectin, collagen and vitronectin. Through this activity, they mediate cell adhesion to ECM proteins or to the surfaces of other cells [39]. Generally, an individual integrin may recognize several distinct proteins [25,48].

Tumor cell adhesiveness correlates with the metastatic ability of that tumor [1,40]. Among the integrins, the $\alpha_v\beta_3$ receptor, also known as the vitronectin receptor, has been reported to be involved in tumor cell migration [15,28]. The $\alpha_v\beta_3$ receptors are expressed on metastatic tumor cells, and, their expression is restricted to cells within a vertical growth phase [2]. In addition, the $\alpha_v\beta_3$ integrin is involved in angiogenesis [46]. The $\alpha_v\beta_3$ receptors are expressed abundantly on capillary blood vessels, which are essential to tumor growth during angiogenesis, while they were not detectable on normal blood vessels [6]. Furthermore, antagonists of $\alpha_v\beta_3$ integrin are reported to induce the apoptosis of angiogenic vascular cells [7]. Thus, the $\alpha_v\beta_3$ integrin participates in tumor metastasis and tumor-related angiogenesis. In order to enhance tumor detection and to measure the tumor's metastatic potential, we have developed an $\alpha_v\beta_3$ receptor-imaging agent for use in positron emission tomography (PET).

Several radiolabeled ligands of the $\alpha_v\beta_3$ receptor have recently been developed [5,11,18–21,27,33,34,44], based on the integrins recognition of the Arg-Gly-Asp (RGD) sequence of adhesive proteins [41]. Linear peptides containing RGD sequences have been labeled with ^{99m}Tc, ^{188}Re, ^{90}Y, ^{111}In. In addition, cyclic peptides, which are stronger

Abbreviations: MeVal, methylvaline; FPhe, fluorophenylalanine.

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EXHIBIT B

and more selective inhibitors of cell adhesion compared to linear variants [3], have also been labeled with ^{125}I, ^{111}In and ^{18}F.

Indirect multi-step methods have been reported for the synthesis of fluorine-18-labeled peptide either in a liquid phase [13,32] or in a solid phase [47]. However, these methods require a large fluorinated component for the introduction of fluorine into the peptide. In some cases, these components may alter the nature of the peptide to a large extent. Therefore, we developed a simple direct fluorine-introducing method for peptides containing a phenylalanine residue using an electrophilic reaction. This methodology is also advantageous as it does not alter the net structure and the biochemical nature of the peptide. In this study, we selected cyclo(Arg-Gly-Asp-D-Phe-MeVal) (cyclo(RGDfMeV)) as a substrate for fluorination since this phenylalanine containing peptide has the highest affinity to $\alpha_v\beta_3$ [12]. Subsequently, we evaluated the feasibility of the radiofluorinated peptide as an $\alpha_v\beta_3$ integrin associated tumor imaging agent for PET.

2. Materials and methods

2.1. General

Only reagent grade chemicals were used. All reagents for peptide synthesis were purchased from Watanabe Chemical Industries, Ltd. (Hiroshima, Japan), Nacalai Tesque Inc. (Kyoto, Japan) and Wako Pure Chemical Industries Ltd. (Osaka, Japan). Male BALB/c-*nu/nu* mice were supplied by CLEA Japan, Inc. (Tokyo, Japan) and DLD-1 cells (human colon adenocarcinoma) were purchased from Human Health Science Foundation (Tokyo, Japan). ^1H NMR spectra were recorded using a Bruker AM 600 spectrometer at 600 MHz ^1H frequency in DMSO-d_6. Ion-spray mass spectra were obtained with a Sciex API*III*E triple quadrupole mass spectrometer. An automated gamma counter with a NaI(Tl) detector (COBRA, PACKARD) was used to measure the radioactivity of samples from the animal. The present animal study was approved by the Animal Care and Use Committee of the National Institute for Longevity Sciences.

2.2. Synthesis of the cyclic pentapeptide, cyclo(Arg-Gly-Asp-D-Phe-MeVal) [12]

A protected peptide, cyclo[Arg(Mts)-Gly-Asp(OBzl)-D-Phe-MeVal], was constructed using a similar Boc-based solution phase synthetic strategy with that described for the preparation of the pseudopeptide, cyclo(Arg-Gly-Asp-D-Phe-Ψ[(*E*)CH=CH]-Val) [37]. The protected peptide was treated with 1M TMSBr-thioanisole-TFA in the presence of *m*-cresol (room temperature, overnight) to remove the protecting groups. Usual workup and purification by reversed phase HPLC (Cosmosil 5C18-ARII column, 20 × 250 mm, Nacalai Tesque Inc., Japan) eluted with 17% CH_3CN in water yielded the title compound, which was fully characterized by mass-spectrometry and amino acid analysis of the 6N HCl hydrolysate.

2.3. Synthesis of the fluorinated cyclic pentapeptide, cyclo(Arg-Gly-Asp-D-FPhe-MeVal)

After converting *p*-nitrophenylcarbonate Wang resin [14,16] to the hydrazino linker by treatment with excess hydrazine hydrate, the resin-bound peptide was constructed using the standard Fmoc-based solid phase synthesis. Amino acid coupling was performed using PyBrop-DIEA for MeVal and D-FPhe and DIC-HOBt for others. After deprotection by treating with 1M TMSBr-thioanisole/TFA in the presence of *m*-cresol (0°C, 2 hr), the mixture was purified by reversed phase HPLC (Cosmosil 5C18-ARII column, 20 × 250 mm, Nacalai Tesque Inc., Japan) eluted with 14% CH_3CN in 0.1% TFA aq. to give the acyclic peptidehydrazide (H-Asp-D-FPhe-Val-Arg-Gly-$NHNH_2$). The peptidehydrazide was cyclized by the azide method [22] under high dilution condition in DMF. Subsequent HPLC purification was performed using the same column as above with gradient elution using CH_3CN-H_2O (84:16 to 80:20) in 0.1% TFA to yield the cyclic peptides. Three cyclic peptides, each containing 2-F, 3-F and 4-F substituted D-Phe regioisomers, were prepared by the same method, and were characterized by ^1H-NMR, mass-spectrometry and amino acid analysis of the 6N HCl hydrolysate.

2.4. Direct radiofluorination of the peptide

$[^{18}F]F_2$ was produced by deuteron irradiation of ^{20}Ne gas containing a small amount of carrier F_2 (0.15%), and was subsequently converted to $[^{18}F]$AcOF by passing through a $CH_3COONa{\cdot}3H_2O$ column. $[^{18}F]$AcOF was bubbled into 2 mL of trifluoroacetic acid (TFA) containing various amount of cyclo(Arg-Gly-Asp-D-Phe-MeVal) (cyclo(RGDfMeV)) at a flow rate of 100 mL/min at room temperature. After the removal of TFA with N_2 gas flow, the residue was dissolved in 4 mL of 25% acetonitrile in water and separated by HPLC using a reverse-phase column (CAPCELL PAK C-18 UG120, 20 × 250 mm, Shiseido, Japan) eluted with a mixture of CH_3CN and 0.1% TFA in water (25:75) at a flow rate of 10 mL/min. The HPLC profiles are shown in Fig. 2. For animal studies, two radioactive fractions containing ^{18}F-labeled peptides, fraction 1 and fraction 2, were collected. After evaporation of the mobile phase, the residue was dissolved in 5% ethanol-saline and filtered (MILLIPORE VENTED MILLEX-GS 0.22 μm). The specific activity was measured by comparing the UV peak area (220 nm) of an unlabeled standard.

2.5. Integrin-binding assay

Compounds were evaluated for their inhibitory activities in $\alpha_v\beta_3$ and $\alpha_{IIb}\beta_3$-ELISA (enzyme linked immunosorbent

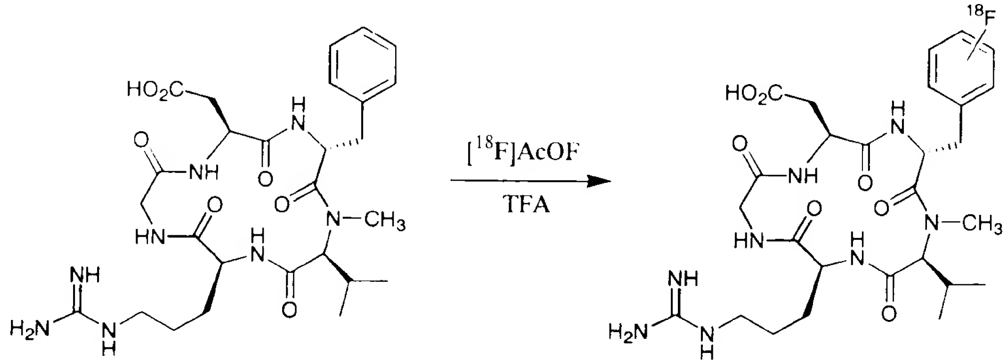

Fig. 1. Radiosynthetic scheme of [^{18}F]fluorinated cyclo(RGDfMeV) by direct fluorination with [^{18}F]AcOF.

assay). $\alpha_v\beta_3$ [38] was purified from human placenta, using RGDSPK-sepharose CL-4B chromatography, followed by mono Q chromatography (Pharmacia). $\alpha_{IIb}\beta_3$ [38] was purified from human platelet by RGDSPK-sepharose CL-4B. $\alpha_v\beta_3$ and $\alpha_{IIb}\beta_3$ binding assays were performed according to the modified method of Kouns et al. [30]. EIA plates (Nunc, Denmark) were coated with $\alpha_v\beta_3$ or $\alpha_{IIb}\beta_3$, and blocked with bovine serum albumin. In each reaction, the reaction mixture (20 mM Tris-HCl, 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, pH 7.4, 100 μl) including vitronectin (Calbiochem, USA) or fibrinogen, added to the receptor-coated plate was incubated for 4 hrs at 25°C. Thereafter the ligand binding was measured using anti-vitronectin rabbit antibody (Calbiochem, USA) and peroxidase-conjugated anti-rabbit IgG antibody (Capell) for $\alpha_v\beta_3$, or peroxidase-conjugated anti-fibrinogen antibody (Capell) for $\alpha_{IIb}\beta_3$, and 2, 2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (Sigma, USA) as the substrate of peroxidase. The IC_{50} values were determined from measurement of absorbance at 415 nm.

2.6. Tissue distribution

DLD-1 (human colon adenocarcinoma) bearing male BALB/c-*nu/nu* mice were prepared by the subcutaneous injection of the tumor cells under the skin on their backs. Two weeks after the dissemination, the mice were used for the biodistribution study. The mice were injected with 0.37 MBq of each ^{18}F-labeled peptide through a tail vein. The animals were killed and dissected at 5 and 60 min after the injection of the radiotracers. For *in vivo* blocking study, the mice were treated with 8.6 mg/kg cyclo(RGDfMeV) intravenously, simultaneously with the [^{18}F]2-F-cycloRGD injection. Then the mice were killed 60 min after the injection. The dissected tissues were weighed and the radioactivity was measured by the automated gamma counter. Data were calculated as the percent injected dose per gram of tissue.

3. Results

3.1. Radiosynthesis

The labeling of cyclo(RGDfMeV) was accomplished by direct fluorination using [^{18}F]AcOF (Fig. 1). As shown in radiochromatograms of the reaction mixture (Fig. 2), four radioactive fractions containing [^{18}F]fluorinated cyclo-(RGDfMeV) were observed. Mass spectroscopy indicated that fraction 1 and 2 were mono-fluorinated isomers, with a small amount of contaminant di-fluorinated congener in fraction 2, while fraction 3 and 4 were di-fluorinated isomers. The HPLC retention time of fraction 1 was consistent with that of a sample of authentic cyclo[Arg-Gly-Asp-D-(2-F)Phe-MeVal] (2-F-cycloRGD). Furthermore, ^{1}H-NMR analysis identified fraction 1 as 2-F-cycloRGD (Fig. 3). The 3- and 4- fluorinated isomers showed similar retention times in HPLC, which were equivalent to that of fraction 2. ^{1}H-NMR indicated that fraction 2 was comprised mainly of 4-fluorinated isomer, although a slight amount of 3-fluorinated and di-fluorinated peptides may also be present (Fig. 3). Since the separation of the isomers in fraction 2 was difficult, we conducted the animal studies using the mixture. The specific activity of fraction 1, [^{18}F]2-F-cycloRGD was 32.8 GBq/mmol at the end of synthesis (EOS). The average time for synthesis was 29 min from the end of bombardment (EOB), excluding the purification step. Table 1 shows the results of direct fluorination under different reaction conditions. In each condition, the mono-fluorinated isomers were predominately produced. A small amount of di-fluorinated peptides were also observed and the proportion of di-fluorinated isomer tended to increase with increasing AcOF. However, it should be noted that even in the case of [^{18}F]AcOF to cyclo(RGDfMeV) ratio raised to 10, the substrate peptide remained in the reaction mixture.

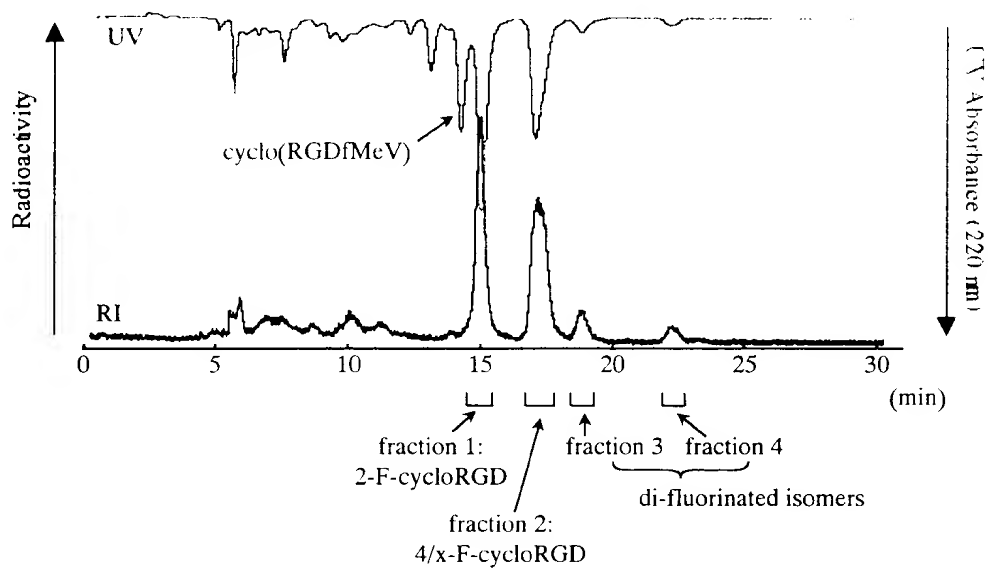

Fig. 2. Reverse-phase HPLC profiles in [18]F-radiolabelling.

3.2. Integrin-binding assay

In order to estimate the affinity and selectivity of the binding of the fluorinated cyclic peptides to the $\alpha_v\beta_3$ receptor, an *in vitro* assay was performed using the immobilized $\alpha_v\beta_3$ or $\alpha_{IIb}\beta_3$ receptors. The affinities were compared to that of the linear standard peptide, RGDS. All of the fluorinated peptides showed almost the same affinity for the receptors as the non-fluorinated peptide, cyclo(RGDfMeV) as shown in Table 2. Furthermore, all of the peptides examined showed more than 100-fold selectivity to the $\alpha_v\beta_3$ integrin.

3.3. Biodistribution studies

Tissue distributions of the labeled peptides, [^{18}F]2-F-cycloRGD (fraction 1) and [^{18}F]4/x-F-cycloRGD (fraction 2, mixture of [^{18}F]4-F-cycloRGD and slight amount of other peptides) are summarized in Table 3. The highest uptake was found in the liver, followed by the kidney. The accumulated radioactivity in these organs decreased with time, while it gradually increased in the intestine. Both of the investigated tracers were also accumulated to tumor. The accumulated activity in tumor was remained rather longer in [^{18}F]4/x-F-cycloRGD comparing to [^{18}F]2-F-cycloRGD. The smaller liver and kidney uptakes and faster clearance from the intestine of [^{18}F]4/x-F-cycloRGD were also observed.

The resulted tumor to organ ratios are shown in Table 4. The tumor to blood ratios were increased with time for both [^{18}F]2-F-cycloRGD and [^{18}F]4/x-F-cycloRGD, and they reached higher values, 5.28 and 4.34 at 60 min postinjections, respectively. High tumor to muscle ratios were also observed in both tracers and they increased with time. In contrast, the tumor to bone ratios were low and remained constant, especially in [^{18}F]4/x-F-cycloRGD.

By cyclo(RGDfMeV) co-treatment, 53% of the radioactivity accumulation to the tumor was significantly decreased. In contrast, no reduction was observed in muscle (Table 5). Significant reductions of the uptake in spleen, pancreas, heart as well as lung were observed.

4. Discussion

The ^{18}F-labeling of proteins or peptides are principally performed by treating the protein with activated ^{18}F-labeled components [49], such as [^{18}F]fluorobenzoate derivatives [32]. However, these large substituent groups may alter tertiary structure of peptides, which could influence their biochemical function. Recently, a nonradioactive direct electrophilic fluorination method for tyrosine-containing peptide using acetyl hypofluorite (AcOF) as an electrophile has been reported [31]. [^{18}F]Acetyl hypofluorite is often employed in the ^{18}F-labeling of amino acids and amino acid derivatives that contain an aromatic ring [8,10]. Taking these evidences in account, the aromatic ring of tyrosine or phenylalanine of peptides may be a target for electrophilic radiofluorination. In the present study, by applying this direct fluorination technique, we successfully introduced ^{18}F into a peptide. Consequently, these fluorinated peptides showed high affinities and high selectivities to the $\alpha_v\beta_3$ integrin. Their binding profiles were similar to that of the non-fluorinated peptide, cyclo(RGDfMeV), therefore, the introduction of F atom in this cyclic peptide seems to hardly affect its receptor binding.

Accordingly, this labeling method was found effective as it causes less steric hindrance especially for peptides whose

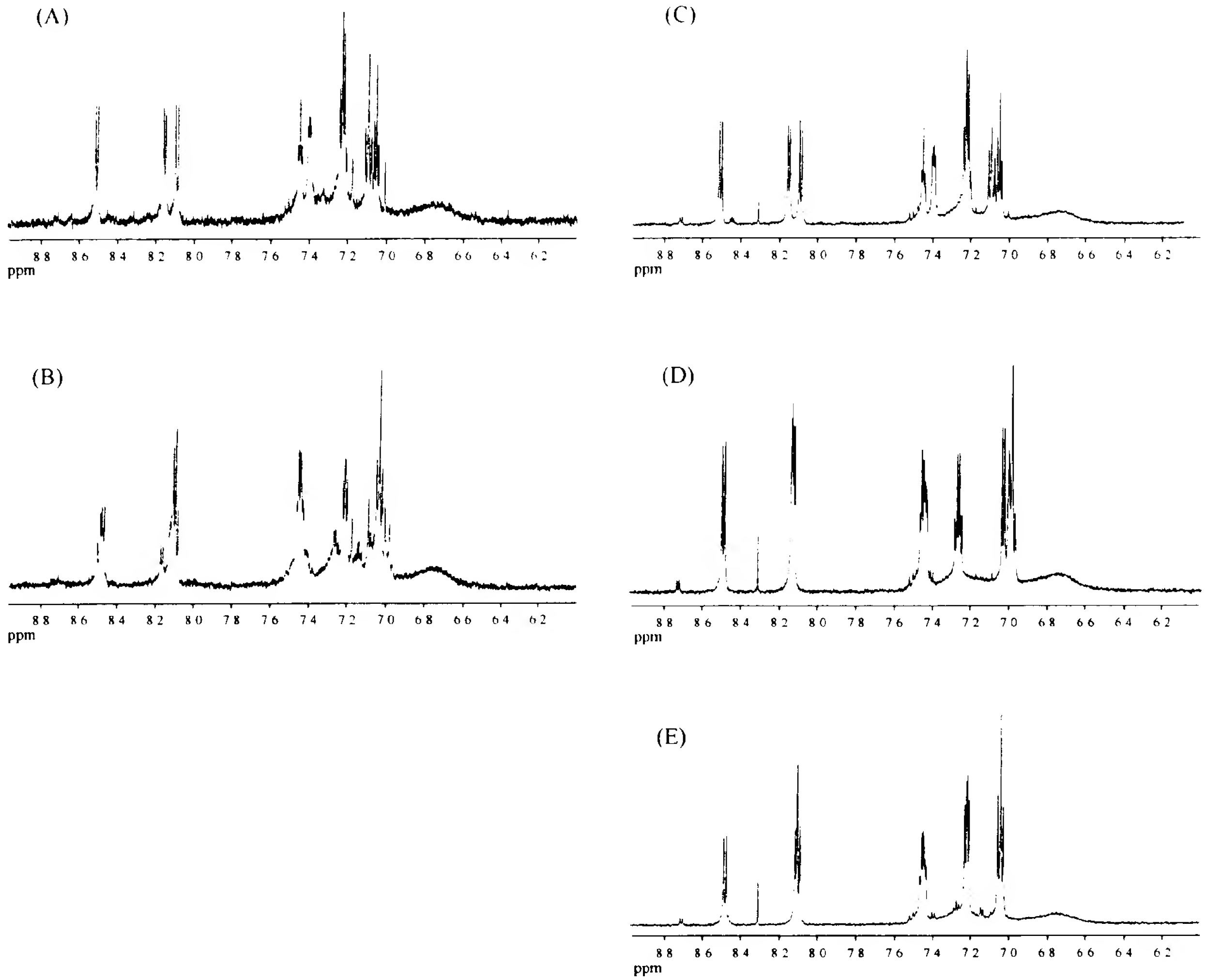

Fig. 3. 1H-NMR spectra of fraction 1 (A), fraction 2 (B), and authentic samples of 2-F-cycloRGD (C), 3-F-cycloRGD (D) and 4-F-cycloRGD (E) around 6 ppm to 9 ppm, which are ascribed to aromatic proton. Spectra were obtained at 600 MHz in DMSO-d_6.

receptor recognition is strict for its structure. Moreover, this method is effective for the peptides whose *N*-terminal structure is important for the receptor recognition. However, since methionine, cysteine and tryptophan can be oxidized by AcOF [31], this procedure may not be applicable when the peptide contains these oxidation-sensitive amino acids.

We preliminary studied the effect of molecular ratio of the substrate peptide and fluorinating agent, [^{18}F]AcOF. In several labeling conditions using different cyclo(RGD-fMeV) to [^{18}F]AcOF ratios, peptides that were mono-fluorinated in their phenylalanine residue were primarily obtained, while di-fluorinated peptides were produced in small amounts. Previously, we reported that increasing the ratio of [^{18}F]AcOF to the fluorination substrate leads to a higher yield of di-fluorinated isomer on the aromatic ring in preparing [^{18}F]fluoro-L-DOPA [17]. This was also observed in the present study, in that when a smaller amount of the

Table 1
Results of direct ^{18}F-Fluorination with [^{18}F]AcOF

	cyclo(RGDfMeV) to [^{18}F]AcOF ratio[§]		
	0.5	0.25	0.1
Trapped radioactivity (%)[†]	N.D.	56.2	56.8
Incorporated radioactivity (%)[‡]	N.D.	68.5	31.0
fraction 1 (2-F) %*	32.8	27.4	24.7
fraction 2 (mainly 4-F) %*	30.3	29.3	30.1
fraction 3 (di-F) %*	1.6	5.5	7.0
fraction 4 (di-F) %*	N.D.	2.6	3.3

* The radiochemical yield based on the injected radioactivity to RP-HPLC.

† Percentage based on the total radioactivity recovered as [^{18}F]AcOF.

‡ Percentage of residual radioactivity after the solvent evaporation against the trapped radioactivity.

§ Molar ratio of cyclo(RGDfMeV) to [^{18}F]AcOF.

N.D.: not determined.

Table 2
The inhibition ability of the peptides for vitronectin binding to $\alpha_v\beta_3$ and fibrinogen binding to $\alpha_{IIb}\beta_3$ (n = 2)

Peptide	$\alpha_v\beta_3$ IC$_{50}$ (nM)	$\alpha_v\beta_3$ Q*	$\alpha_{IIb}\beta_3$ IC$_{50}$ (nM)	$\alpha_{IIb}\beta_3$ Q*	$\alpha_v\beta_3$ vs. $\alpha_{IIb}\beta_3$ †
RGDS (control peptide)	89, 41	1.0	270, 290	1.0	3, 7
cyclo(RGDfMeV)	2.3	39‡	260‡	1.0‡	120
fraction 1	1.6	54‡	190‡	1.5‡	110
fraction 2	2.0	45‡	200‡	1.4‡	100
2-F-cycloRGD	1.0	41§	180	1.6	180
3-F-cycloRGD	0.76	54§	270	1.1§	350
4-F-cycloRGD	1.4	30§	290	1.0§	210

* Q = IC$_{50}$[peptide]/IC$_{50}$[RGDS].
† Data are presented as IC$_{50}$[$\alpha_v\beta_3$] to IC$_{50}$[$\alpha_{IIb}\beta_3$] ratio.
‡ The former value of IC$_{50}$[RGDS] was employed for calculation.
§ The latter value of IC$_{50}$[RGDS] was employed for calculation.

substrate is used, more di-fluorinated peptides are produced. However, in the case of [^{18}F]AcOF to cyclo(RGDfMeV) ratio was 10, the yield of di-fluorinated compounds was only 10.3%, while that of mono-fluorinated compounds was 54.8%. Moreover, even with a smaller amount of cyclo (RGDfMeV), the substrate peptide remained in the reaction mixture. These results suggest that di-fluorination of the peptide with [^{18}F]AcOF is less likely to occur when compared to mono-fluorination.

Coenen *et al.* [9] reported no formation of [^{18}F]3-F-phenylalanine using the [^{18}F]F_2 method. Similarly, the amount of [^{18}F]3-F substituent produced in our peptide labeling study was small. Previously, Murakami et al. [35] reported that [^{18}F]2-F-phenylalanine is more likely to be produced than [^{18}F]3/4-F-phenylalanines in an electrophilic fluorination reaction with [^{18}F]AcOF, although these isomers were unidentified. However, in our study, the radiochemical yields of [^{18}F]2-F and [^{18}F]4-F isomer were similar. The difference between these studies is in the reaction temperature. While Murakami et al. used a reaction temperature of 0°C, we performed our reactions at room temperature. This different isomeric preference may also be attributed to either the reaction condition or the properties of substrates.

The ^{18}F-labeled peptides thus obtained were distributed to a certain extent into tumors in adenocarcinoma-bearing nude mice. This accumulation is comparable to that of [^{18}F]FDG in adenocarcinoma-bearing mice [45], which is a common radiotracer to detect tumors using PET. The ^{18}F-labeled peptides were highly accumulated in the liver, and the radioactivity decreased with time. This observation was

Table 4
Tumor to organ uptake ratios of ^{18}F-labeled cyclo(RGDfMeV) in adenocarcinoma-bearing nude mice

	Time after injection (min)			
	2-F-cycloRGD (fraction 1)		4/x-F-cycloRGD (fraction 2)	
	5*	60†	5*	60‡
Tumor to blood	0.78 ± 0.08	5.28 ± 0.37	0.83 ± 0.12	4.34 ± 0.35
Tumor to muscle	1.78 ± 0.28	3.06 ± 1.16	2.92 ± 0.52	4.05 ± 1.35
Tumor to bone	1.08 ± 0.37	1.59 ± 0.44	1.31 ± 0.20	1.40 ± 0.29

Expressed as the mean ± S.D.
* n = 3.
† n = 5.
‡ n = 4.

Table 3
Biodistribution of radioactivity after administration of ^{18}F-labeled cyclo(RGDfMeV) in adenocarcinoma-bearing nude mice

Organs	Time after injection (min)			
	2-F-cycloRGD (fraction 1)		4/x-F-cycloRGD (fraction 2)	
	5*	60†	5*	60‡
Blood	2.05 ± 0.19	0.17 ± 0.02	2.11 ± 0.26	0.24 ± 0.04
Intestine	2.24 ± 0.84	13.1 ± 9.11	2.36 ± 0.14	8.04 ± 6.38
Liver	46.4 ± 4.59	14.5 ± 2.22	39.7 ± 3.15	11.8 ± 0.53
Kidney	15.1 ± 1.19	3.57 ± 0.97	14.2 ± 0.56	2.27 ± 0.37
Stomach	2.54 ± 0.34	2.53 ± 1.33	2.14 ± 0.24	1.51 ± 0.37
Spleen	1.78 ± 0.18	1.18 ± 0.36	1.91 ± 0.10	1.11 ± 0.36
Pancreas	0.73 ± 0.18	0.41 ± 0.32	0.74 ± 0.08	0.99 ± 0.55
Lung	2.73 ± 0.45	0.49 ± 0.16	2.81 ± 0.20	0.98 ± 0.76
Heart	0.99 ± 0.20	0.21 ± 0.03	1.00 ± 0.21	0.58 ± 0.40
Muscle	0.92 ± 0.23	0.33 ± 0.15	0.60 ± 0.09	0.29 ± 0.13
Bone	1.54 ± 0.30	0.59 ± 0.18	1.33 ± 0.13	0.77 ± 0.20
Brain	0.12 ± 0.002	0.04 ± 0.004	0.13 ± 0.01	0.06 ± 0.01
Tumor	1.61 ± 0.30	0.88 ± 0.06	1.73 ± 0.10	1.03 ± 0.09

Expressed as percent of injected dose per gram of organ as the mean ± S.D.
* n = 3.
† n = 5.
‡ n = 4.

Table 5
The effect of cyclo(RGDfMeV) co-treatment in the accumulation of [^{18}F]2-F-cycloRGD in adenocarcinoma-bearing nude mice, 60 min after the radiotracer injection

Organs	% of saline treatment
Blood	118 ± 50
Intestine	92.0 ± 9.1
Liver	87.1 ± 22.4
Kidney	120 ± 47
Stomach	118 ± 108
Spleen	27.8 ± 7.4‡
Pancreas	22.5 ± 12.0†
Lung	46.7 ± 15.9†
Heart	36.7 ± 14.0†
Muscle	83.3 ± 64.3
Bone	59.8 ± 42.6
Brain	77.5 ± 27.3
Tumor	53.1 ± 8.7†

Expressed as percent of saline treated study as the mean ± S.D. based on percent of injected dose per gram of organ.
n = 3 for cyclo(RGDfMeV) treatment and n = 4 for saline control study.
Significant reduction from the control experiment was observed by *t* test with Welch's correction.
† $p < 0.05$.
‡ $p < 0.01$.

accompanied by a gradual increase in radioactivity in the intestine. This result suggests that these peptides are metabolized in the liver, followed by the biliary excretion of the radioactive metabolites.

The tumor to blood ratios of these labeled peptides increased with time, reaching 5.28 ([^{18}F]2-F-cycloRGD) and 4.34 ([^{18}F]4/x-F-cycloRGD) at 60 min postinjection. These values are comparable with that observed for L-[methyl-^{11}C]methionine, 4.72 [26] and higher than that observed for [^{67}Ga]Ga citrate, 2.03 (the value of 24hr postinjection) [45], although they are lower than that observed for [^{18}F]FDG [45].

The co-injection of cyclo(RGDfMeV) significantly reduced the tumor uptake in 53% of non-treated group for [^{18}F]2-F-cycloRGD. Uptake of radioactivity in the spleen and lung, in which the expression of $\alpha_v\beta_3$ integrin has been reported [42,43], were also significantly reduced by the cyclo(RGDfMeV) treatment. Furthermore, the accumulation to the muscle, in which no $\alpha_v\beta_3$ integrin expression is observed, did not decline with the cyclo(RGDfMeV) treatment. These findings suggest that [^{18}F]2-F-cycloRGD binds to $\alpha_v\beta_3$ receptor in tumor following injection.

The radioactivity was taken by the bone to some degree, but in contrast to the tumor to blood and tumor to muscle ratios, the tumor to bone ratio remained constant during the time examined. Since osteocrasts express $\alpha_v\beta_3$ integrins [36] and the bone uptake of radioactivity (^{18}F$^-$) resulting from the defluorination will not decline with time, this bone radioactivity may not be due to defluorination and reflect $\alpha_v\beta_3$ specific binding. Actually, there is a tendency that the radioactivity in the bone decreased with cyclo(RGDfMeV) treatment. Furthermore, the radioactivity uptake in the pancreas and heart was reduced by the cyclo(RGDfMeV) treatment. This finding may also reflect the binding of the ^{18}F-labeled peptide to $\alpha_v\beta_3$ receptor in these organs, although further studies are required to determine the expression of $\alpha_v\beta_3$ integrin in these organs.

Biodistribution studies of [^{18}F]2-F-cycloRGD also indicated that there are some problems to be overcome. First, since the synthesis of [^{18}F]AcOF is achieved with a carrier fluorine [29] and specific activity of the labeled peptide synthesized is low in this study, uptake of radioactivity in the tumor may be inhibited by the cold ligand if the ^{18}F-labeled peptide binds to $\alpha_v\beta_3$ receptor in tumor. Recently, the study to elevate the specific activity of the [^{18}F]F_2 has been reported [4]. The application of [^{18}F]AcOF with higher specific activity to the present peptide preparation may result in the improved tumor uptake. Second, the level of radioactivity in the liver, intestine and kidney was high and therefore, at the present status, these peptides may not be effective as a practical tumor detecting agent in the body structure near these organs. Recently, several analogues of RGD-containing peptides labeled with ^{125}I, ^{99m}Tc, ^{111}In, ^{188}Re, ^{90}Y and ^{18}F were reported [5,11,18–21,27,33,35,44]. Of these labeled peptides, [^{18}F]galacto-RGD [21] is a candidate as a PET imaging agent. This glycosylated cyclopeptide is labeled using 4-nitrophenyl-2-[^{18}F]fluoropropionate *via* [^{18}F]fluoroacylation. The tumor accumulation and tumor to blood ratio of both [^{18}F]2-F-cycloRGD and [^{18}F]4/x-F-cycloRGD used in this study are comparable to those of [^{18}F]galacto-RGD. However, our peptides persisted for a longer period in the liver than did [^{18}F]galacto-RGD. The effectiveness of the hydrophilic glycosyl group in reducing the radioactivity accumulation in the liver has already been confirmed by iodinated RGD-containing cyclo peptides [19,20]. In a similar manner as these glycopeptides, the liver accumulations of [^{18}F]2-F-cycloRGD and [^{18}F]4/x-F-cycloRGD may be diminished by the introduction of a hydrophilic substituent.

In this study, we successfully labeled phenylalanine containing peptide by a direct electrophilic fluorination method using [^{18}F]AcOF. The resulting radiofluorinated $\alpha_v\beta_3$ integrin ligands, [^{18}F]2-F-cycloRGD and [^{18}F]4/x-F-cycloRGD, had a similar affinity to the non-fluorinated peptide, cyclo(RGDfMeV). This fairly simple labeling method is useful for fluorinating the peptides. We could see the $\alpha_v\beta_3$ receptor specific accumulation in the tumor, however, the absolute accumulated amount was not efficient for tumor detection in the present status. The tumor radioactivity level is not efficiently high compared to other organs, like liver, intestine and kidney. Adequate drug design to diminish the non-specific accumulation and higher specific activity to increase the receptor specific fraction in the tumor are required for this peptidic tracer. These [^{18}F]fluorinated cyclic peptides may become informative scaffolds of $\alpha_v\beta_3$ integrin positive tumor-imaging agent. By imaging $\alpha_v\beta_3$ receptor expression, we should obtain valuable information concerning tumor status, such as the metastatic ability of the tumor, which is not available through the commonly used tumor imaging agent, [^{18}F]FDG.

Acknowledgments

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